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CARM1 promotes adipocyte differentiation by coactivating PPARγ

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The coactivator-associated arginine methyltransferase 1 (CARM1) is recruited to gene promoters by many transcription factors. To identify new pathways that use CARM1, we carried out a comprehensive transcriptome analysis of CARM1-knockout embryos. By using complementary DNA microarrays and serial analysis of gene expression, we identified various genes involved in lipid metabolism that were underrepresented in CARM1-knockout embryos, indicating an important role for this coactivator in adipose tissue biology. We also observed that the amount of brown fat in CARM1-knockout embryos is reduced. Furthermore, cells lacking CARM1 have a severely curtailed potential to differentiate into mature adipocytes. Reporter experiments and chromatin immunoprecipitation analysis show that CARM1 regulates these processes by acting as a coactivator for peroxisome proliferator-activated receptor gamma (PPARγ). Together, these results show that CARM1 promotes adipocyte differentiation by coactivating PPARγ-mediated transcription and thus might be important in energy balance.

Keywords: CARM1; adipocyte; coactivator; PPARγ

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INTRODUCTION

Arginine methylation is a common post-translational modification that regulates several cellular processes, including transcription, splicing and translation (Bedford & Richard, 2005). There are nine mammalian protein arginine methyltransferases (PRMTs), which are divided into two classes: Type I enzymes that catalyse the formation of ω-N^G, N^G-asymmetrical dimethylarginine (aDMA), and Type II enzymes that catalyse ω-N^G, N'^G-symmetrical dimethylarginine formation (sDMA; Clarke & Tamanoi, 2006). PRMT5 is the main Type II enzyme in mammals and its sDMA activity is primarily associated with transcriptional repression (Pal *et al*, 2003). Conversely, PRMT1 and coactivator-associated arginine methyltransferase 1 (CARM1) generate aDMA residues, and function as transcriptional coactivators. Indeed, PRMT1 and CARM1 methylate distinct substrates and function synergistically in reporter assays (Koh *et al*, 2002).

CARM1 was identified as a binding partner for the p160 steroid receptor coactivator, glutamate receptor interacting protein 1 (GRIP1; Chen *et al*, 1999). This recruitment of a secondary coactivator (CARM1) by a primary coactivator (GRIP1) results in the methylation of histone H3 at Arg 17 (H3R17), the histone acetyltransferases p300/CBP (Lee *et al*, 2005), the p160 steroid receptor coactivator SRC3 (Feng *et al*, 2006; Naeem *et al*, 2007) and a cohort of splicing factors including CA150 (Cheng *et al*, 2007). CARM1 functions as a coactivator for many nuclear receptors, such as oestrogen receptor, androgen receptor, thyroid receptor and farnesoid X-receptor (FXR; Chen *et al*, 1999; Ananthanarayanan *et al*, 2004). It also coactivates other transcription factors such as myocyte enhancer factor 2C (MEF2C), β-catenin, p53, nuclear factor (NF)-κB (reviewed by Bedford & Richard, 2005) and the cAMP-responsive element-binding factor (Krones-Herzig *et al*, 2006). Thus, CARM1 casts a 'cloud' of methylation over many active promoter regions. A biological consequence of this methylation is the generation of docking motifs for tudor domain-containing proteins. Indeed, CA150—a protein that integrates transcription to splicing—methylation by CARM1, facilitates a tudor domain-mediated interaction with the spinal muscular atrophy protein SMN, and this event promotes exon skipping (Cheng *et al*, 2007).

CARM1-knockout embryos are reduced in size, die perinataly and show a partial block in the development of T cells (Yadav *et al*, 2003; Kim *et al*, 2004). To identify primary *in vivo* pathways in which CARM1 functions as a transcriptional coactivator, we undertook a transcriptome analysis of wild-type and knockout

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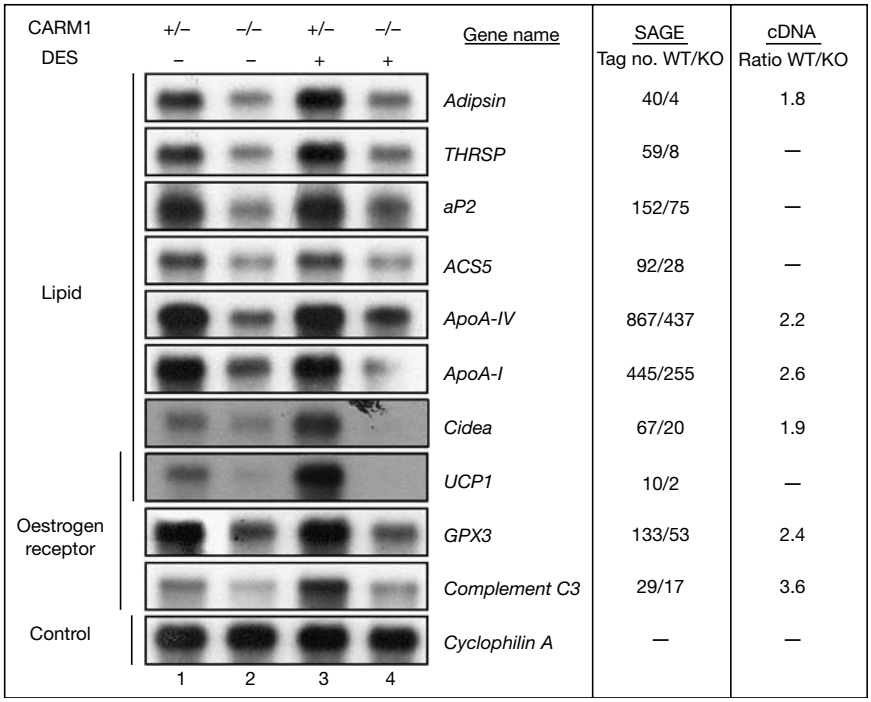

Fig 1 | Transcriptome analysis showed changes in oestrogen-regulated and lipid-associated transcripts. Northern blot analysis of transcripts downregulated in CARM1-knockout embryos (left). Embryonic day 18.5 heterozygous and knockout embryos with (+) or without (–) treatment (DES) were used to isolate messenger RNA. The number of tags obtained from SAGE analysis are listed for WT and KO embryos. The fold change in transcript levels obtained from complementary DNA microarray analysis is listed as ratio WT/KO. CARM1, coactivator-associated arginine methyltransferase 1; DES, diethylstibestrol; KO, CARM1-knockout embryos; SAGE, serial analysis of gene expression; WT, wild-type embryo.

embryonic day 18.5 (E18.5) embryos. This analysis revealed a previously unreported function for CARM1 in the process of adipose tissue development.

RESULTS

CARM1 controls expression of adipogenic transcripts

By using complementary DNA microarray and serial analysis of gene expression (SAGE) techniques, we screened for genes requiring CARM1 to augment their expression. cDNA microarrays are sensitive but limited to analysis of the genes on the array, whereas SAGE is not as sensitive but allows the analysis of all expressed genes. SAGE libraries were generated by using messenger RNA obtained from E18.5 embryos. Two SAGE libraries were sequenced: CARM1 wild-type E18.5 embryos (+diethylstibestrol (DES)) and CARM1-knockout E18.5 embryos (+DES). These libraries are publicly accessible at CGAP (http://cgap.nci.nih.gov/SAGE). A comparison of the SAGE tag libraries of wild-type and knockout embryos identified several transcripts that were significantly downregulated in CARM1-knockout embryos (Fig 1, middle column). In parallel, transcriptome analysis using cDNA microarray was carried out with mRNA isolated from E18.5 embryos. Clear changes in the gene expression profiles were observed for several similar transcripts identified by SAGE (Fig 1, right column). The effects of a CARM1-null genotype on the expression of specific genes was confirmed by northern blot analysis (Fig 1, left column).

Predictably, several oestrogen-responsive genes showed reduced expression in the absence of CARM1. These included complement C3 (Sundstrom *et al*, 1989), glutathione peroxidase 3 (Waters *et al*, 2001) and uncoupling proteins (UCP-1; Pedersen *et al*, 2001). In addition, DES treatment upregulated complement C3, CIDEA (cell death-inducing DFFA (DNA fragmentation factor α)-like effector A) and UCP-1 in the wild type (Fig 1, lanes 1 and 3). CARM1 also acts as a coactivator of the thyroid hormone receptor in reporter assays (Chen *et al*, 1999) and we see a strong dependency on CARM1 for normal expression of thyroid hormone-responsive spot (THRSP) 14 (Cunningham *et al*, 1998).

Unexpectedly, we found a cadre of downregulated genes that are involved in adipogenesis. These include the following proteins: THRSP, which is expressed in lipogenic tissues and is required for *de novo* lipogenesis in the lactating mammary gland (Zhu *et al*, 2005); adipocyte fatty acid-binding protein (FABP4/aP2); acyl-CoA synthetase 5 (ACS5); adipsin (ADN); apolipoprotein A-I (APOA-I); and APOA-IV. ADN and the classic peroxisome proliferator-activated receptor gamma (PPARγ)-responsive gene aP2 are important for adipocyte differentiation, whereas APOA-IV and APOA-I are important for lipid homeostasis (Tontonoz *et al*, 1994; Yu *et al*, 2003). Both UCP1 and CIDEA are abundantly expressed in brown fat (Lin & Li, 2004; Porter, 2006) and markedly downregulated in CARM1-knockout embryos. Recently, Krones-Herzig *et al* (2006) showed that the expression of gluconeogenic genes, *PEPCK* and *G6Pase*, was largely dependent on CARM1. Consistent with their results, we saw a decrease in the number of

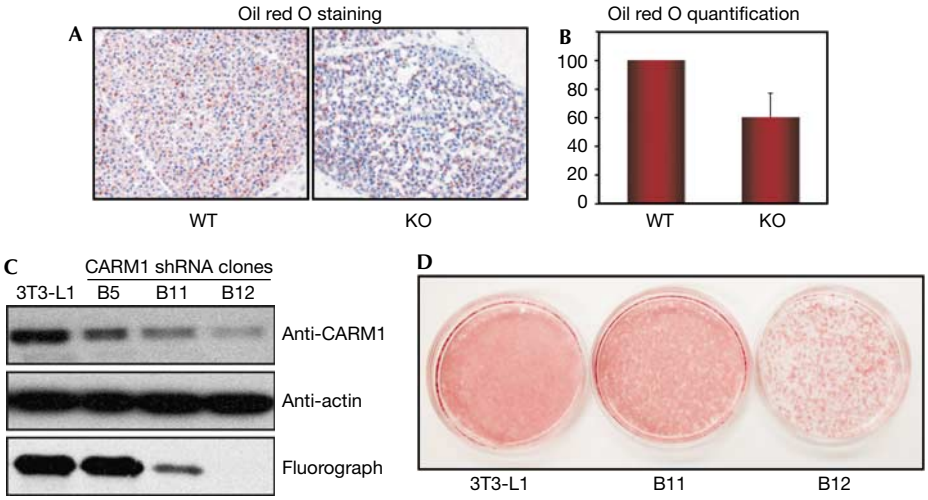

Fig 2 | CARM1 regulates adipose development. (**A**) Levels of interscapular brown adipose tissue in the embryos. Frozen sections of wild-type (WT) and knockout (KO) embryonic day 18.5 (E18.5) embryos were stained with Oil Red O to visualize fat accumulation in the embryos. (**B**) By using a Chromavision imaging system, the intensity of staining of the brown adipose tissue in each genotype was quantitated. For the quantitation, a wild type embryo was paired with a knockout embryo. The knockout staining was quantitated relative to the wild-type staining, which was set as 100 and plotted as mean s.d. Wild-type ($n=4$) and knockout ($n=4$) E18.5 embryos were analysed. (**C**) Knockdown of CARM1 in 3T3-L1 cells shows markedly reduced potential to differentiate into adipocytes. Short hairpin RNA (ShRNA) was used to stably knockdown CARM1 in 3T3-L1 cells. The top panel shows a Western blot with a CARM1 antibody. Western analysis with anti-β-actin acts as a loading control (middle panel). The lower panel is a fluorograph showing CARM1 methyltransferase activity from knockdown cell lysates using GST-PABP1 as a substrate. (**D**) Adipocyte differentiation assay and Oil Red O staining of 3T3-L1 wild-type, and the B11 and B12 CARM1-knockdown cell lines. CARM1, coactivator-associated arginine methyltransferase 1; GST, glutathione-*S*-transferase.

SAGE tags obtained for *PEPCK* (phosphoenolpyruvate carboxykinase; 8 versus 19) and *G6Pase* (4 versus 21) genes when knockout and wild-type embryos were compared. Thus, global transcriptome analysis shows that CARM1 regulates genes important for lipid metabolism.

Brown fat tissue is reduced in CARM1-null embryos

To determine whether CARM1 is required for the normal development of adipose tissue *in vivo*, we sectioned E18.5 embryos and stained them with Oil Red O to visualize adipose tissue. Oil red O stains neutral fat in the lipid droplets in brown adipose tissue. The amount of brown adipose tissue in knockout embryos was reduced when compared with wild-type embryos (as per microscopic observation). Knockout embryos also showed a 40% reduction in lipid accumulation in their brown adipose tissue when compared with the wild-type littermates (Fig 2A,B; supplementary Fig S1 online). This indicates that levels of CARM1 in the embryos greatly affect the amount of brown adipose tissue present. However, loss of CARM1 does not result in the total absence of brown adipose tissue. Thus the function of CARM1 in brown adipose differentiation or maintenance might lie in a semiredundant pathway, possibly with PPARγ coactivator-1α (PGC-1α; see Discussion). Immunohistochemical analysis indicates that the reduction in brown adipose tissue might be due to a partial differentiation block in the absence of CARM1 (supplementary Fig S5 online).

CARM1 knockdown impedes 3T3-L1 differentiation

Differentiation of 3T3-L1 preadipocytes into mature lipid accumulating adipocytes, on treatment with adipogenic stimuli, is an established model to study adipocyte differentiation and the factors affecting this process (Green & Kehinde, 1975). CARM1 is expressed in undifferentiated 3T3-L1 preadipocyte cells and its levels do not increase on differentiation into adipocytes (data not shown). We used short hairpin RNA to knockdown CARM1 in 3T3-L1 cells. Total cell lysates from stable transfectants were used to methylate GST-PABP1—a known substrate of CARM1 (Yadav *et al*, 2003)—in the presence of [^{3}H]AdoMet as the methyl group donor. The levels of CARM1 in the B12 line were greatly reduced and cell extracts were unable to methylate GST-PABP1 (Fig 2C). The B11 line showed reduced CARM1 levels, but lysates had some CARM1 methyltransferase activity. Next we subjected B12 and B11 cells to adipogenic stimuli and compared their differentiation with parental 3T3-L1 cells. CARM1-knockdown cells showed reduced potential to differentiate into mature lipid accumulating adipocytes (Fig 2D). In addition, primary mouse embryonic fibroblasts (MEFs) from Carm1-knockout embryos (E14.5) also showed reduced differentiation potential (supplementary Fig S2 online). Hence, CARM1 promotes differentiation of preadipocytes *in vitro*. However, various factors have been shown to be regulating this process including PPARγ, C/EBPs and FXR, and CARM1 might act as a coactivator for one or more of these factors. Indeed, CARM1 is known to coactivate FXR (Ananthanarayanan *et al*, 2004).

CARM1 is a PPARγ coactivator

CARM1 enhanced the coactivator function of GRIP1 for nuclear hormone receptors (Chen *et al*, 1999), and members of the SRC family have been shown to be the coactivators for the PPARγ-mediated transactivation (Zhu *et al*, 1996). Combinatorial gene

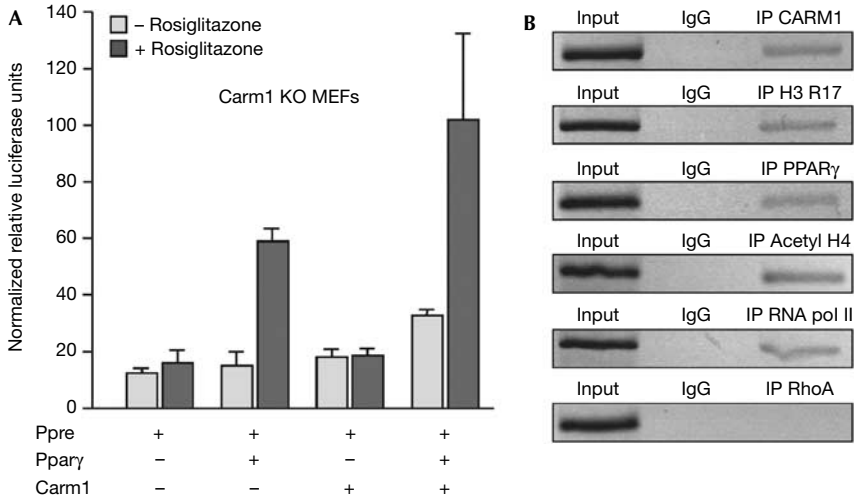

Fig 3 | CARM1 potentiates PPARγ-mediated gene transcription. (**A**) The ability of CARM1 to coactivate PPARγ was assessed in a fatty acyl CoA oxidase PPRE-luciferase assay. Carm1-knockout MEFs were transiently transfected with the indicated plasmids, and renilla as an internal control, using Fugene6. MEFs were treated with either DMSO control (–) or 2 μM (+) rosiglitazone 6 h after transfection. Relative activity of firefly luciferase was normalized to renilla luciferase activity. A mean s.d. of three independent experiments carried out in triplicate is presented. The addition of Carm1 to cells that also express Ppre and Pparγ resulted in a significant increase of luciferase activity (columns 4–8; $P<0.05$). (**B**) CARM1 and PPARγ are recruited to the aP2 gene promoter. 3T3-L1 cells were induced to differentiate and after 5 days cells were subjected to ChIP at the aP2 promoter. The experiment was repeated three times with similar results. CARM1, coactivator-associated arginine methyltransferase 1; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulphoxide; IP, immunoprecipitation; MEF, mouse embryonic fibroblast; PPARγ, peroxisome proliferator-activated receptor gamma.

ablation studies of the SRCs have shown that these coactivators are crucial for energy balance in mice (Picard *et al*, 2002; Wang *et al*, 2006). In addition, several transcripts listed in Fig 1 as regulated by CARM1 are known PPARγ targets. Therefore, we examined whether CARM1 can coactivate PPARγ-dependent transcription. By using Carm1-knockout MEFs and a PPRE reporter, we observed an increase in luciferase activity in the presence of Carm1 (Fig 3A). Carm1 also behaved as a PPARγ coactivator in 293 and 3T3-L1 cell lines (supplementary Fig S3 online). To confirm that Carm1 was recruited to PPARγ-driven promoters, within the context of chromatin, we carried out chromatin immunoprecipitation experiments to show that Carm1 was recruited to the endogenous aP2 promoter, and that Carm1-mediated H3R17 methylation occured at this locus (Fig 3B; supplementary Fig S4 online). Thus, CARM1 is a crucial component of PPARγ receptor transactivation pathway and functions in the adipogenic programme.

DISCUSSION

To our knowledge, the function of PRMTs in adipose tissue has not been previously studied. Differentiation of fibroblast-like cells into adipocytes is highly regulated and a multistep process involving the master regulator PPARγ. Early in this process, C/EBPβ and C/EBPδ are transiently expressed, and lead to the induction of CEBPα and PPARγ. PPARγ and C/EBPα act synergistically to promote the expression of adipocyte-specific genes such as *aP2* (reviewed by Rosen & MacDougald, 2006). Pparγ-knockout mice die at E10 owing to placental defects, and null pups derived by tetraploid rescue lack white and brown adipose tissue (Barak *et al*, 1999). A large family of C2H2 zinc-finger proteins, known as Kruppel-like transcription factors (KLFs), have crucial roles in adipogenesis (Rosen & MacDougald, 2006). In addition, the bile-activated nuclear receptor FXR, which is coactivated by CARM1, has been shown to be involved in adipocyte differentiation (Rizzo *et al*, 2006).

Importantly, members of the p160 family of coactivators have been shown to be important in energy homeostasis. Studies of Grip1$^{-/-}$ and Src1$^{-/-}$ single-knockout mice have shown that these two coactivators control the energy balance between white and brown adipose tissues (Picard *et al*, 2002). Double knockout of Src3 and Src1 led to a block in brown fat development with no lipid accumulation in the tissue, decreased Ucp1 expression and defective adaptive thermogenesis, and hence emphasized the fact that the Src3 and Src1 coactivators are crucial for energy balance (Wang *et al*, 2006). Recently, Src3 was shown to act synergistically with C/EBP transcription factors on Pparγ2 promoters to control its expression in MEFs (Louet *et al*, 2006). Src3-knockout MEFs show severely impaired adipocyte differentiation, and re-expression of Src3 is able to restore this defect. Moreover, Src3-knockout mice show reduced body weight and white adipose tissue mass (Louet *et al*, 2006), and also a reduction in the RNA levels of many selective markers of adipogenesis. Carm1 not only cooperates with Src3 as a coactivator but also methylates Src3 (Feng *et al*, 2006; Naeem *et al*, 2007).

The most extensively studied coactivator of brown fat adipogenesis is PGC1α. It functions by assembling a complex that includes histone acetyltransferases, SRC1 and CBP/p300 leading to chromatin remodelling and promoter activation (Puigserver *et al*, 1999). Pgc1α-knockout mice are viable and

show an inability to maintain core body temperature on cold exposure owing to reduced expression of Ucp1 (Lin *et al*, 2004; Leone *et al*, 2005). Interestingly, CARM1 and PGC1 have similar biological properties: they both function as PPARγ coactivators; they both collaborate with SRCs (Chen *et al*, 1999; Wang *et al*, 2006); and they both fall into a class of coactivators that directly regulates alternative splicing (Monsalve *et al*, 2000; Cheng *et al*, 2007).

The ability of CARM1 to coactivate FXR (Ananthanarayanan *et al*, 2004) and PPARγ (this study), and to behave synergistically with the p160 family of SRCs implicates this arginine methyltransferase at multiple levels of adipose tissue development.

METHODS

cDNA, SAGE and northern blot analysis. Details of SAGE library generation and cDNA analysis are provided as supplementary information online. For northern analysis, embryos were obtained as for SAGE analysis and mRNA was isolated using Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA, USA). A 4 μg portion of the mRNA was run on 1% agarose formaldehyde gel, transferred to membranes and probed. The probes for northern blot analyses were generated by reverse transcription–PCR from mRNA and were sequence validated. Primer sequences for the generation of probes are listed in the supplementary Table I online.

Histology. Embryos (E18.5) were OCT frozen in liquid nitrogen and 4 μm sections were used for staining with OilRed O. Staining was quantitated as described in the supplementary information online.

Short hairpin RNA knockdown of CARM1. Forward and reverse oligonucleotides (see supplementary information online) were annealed and cloned into a pSuper-Puro vector (OligoEngine, Seattle, WA, USA) between *Bgl*II and *Hin*dIII. This vector was used to transfect 3T3-L1 cells and transfectants were selected on 4 μg/ml puromycin (Sigma, St Louis, MO, USA). Resistant colonies were tested for CARM1 expression using a CARM1 antibody (from S.R.).

***In vitro* methyltransferase assay.** Cell lysates were prepared from knockdown cell lines and the *in vitro* methylation assay was carried out as described previously (Yadav *et al*, 2003).

3T3-L1 differentiation assays. 3T3-L1 cells were obtained from ATCC and subjected to a differentiation assay as described previously (Green & Kehinde, 1975; Rizzo *et al*, 2006).

PPARγ luciferase assay. PPRE-CMV-Luc (firefly luciferase) and pSG5-PPARγ were gifts from Dr F. Gonzalez, NCI. phRL-CMV has humanized Renilla luciferase driven by CMV promoter (Promega, Madison, WI, USA). phRL-CMV-renilla was used as a transfection control and experiments were carried out as described previously (Yadav *et al*, 2003).

Chromatin immunoprecipitation assay. 3T3-L1 cells were differentiated as described above. Chromatin immunoprecipitation (ChIP) experiments were carried out using the EZChIP kit (Milipore, Billerica, MA, USA). ChIP was carried out with the following antibodies: H3R17 (Milipore); acetyl H4 and RNA polII (Covance, Denver, PA, USA); PPARγ and RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and CARM1. The primers used to amplify the aP2 gene promoter have been previously described (Yin *et al*, 2006).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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